PATENT
94363CIPDIVDIV

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

**Examiner:** Lisa V. Cook )
**Art Unit:** 1641 )
)
**Applicant:** Minden et al. )
)
**Serial No.:** 10/713,861 )
**Filing Date:** November 14, 2003 )

**Title:**
DIFFERENCE DETECTION METHODS
USING MATCHED MULTIPLE DYES

**DECLARATION OF JONATHAN S. MINDEN UNDER 37 C.F.R. § 1.131**

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Jonathan S. Minden, Ph.D., declare as follows:

1. I am a one of the named co-inventors for the above-captioned application (hereinafter "the subject application").

2. I am a professor in the Biological Sciences Department at Carnegie Mellon University ("CMU") in Pittsburgh, PA and have held that position since June 2007. Prior to that time I was an associate professor at CMU from June 1997 to June 2007 and an assistant professor from January 1991 to June 1997. CMU is the assignee of the subject application.

3. I, along with Alan Waggoner, Ph.D. conceived of the subject matter described and claimed in at least the independent claims of the subject application prior to July 1993.

4. In support of the conception date alleged in paragraph 3 above, I enclose a partially redacted document entitled "Disclosure of Invention" that I, along with Dr. Alan

Waggoner, submitted to the Office of Technology Transfer of CMU. This document, in section 3 and the attached Figures 1 and 2, discloses the invention claimed in the independent claims and most of the dependent claims of the subject application. Information, not critical to the showing of the invention date, is also redacted from this document for confidentiality purposes. The dates that have been redacted from this document are prior to July 1993.

5. In further support of the conception date alleged in paragraph 3 above, I enclose copies of laboratory notebook pages of Christopher Lacenere, a CMU undergraduate student, who, with a graduate student, was working at my direction and under my supervision. The dates that have been redacted from this document are prior to July 1993. The pages show that the students were synthesizing Cy5 and Cy3 dyes, two of the matched multiple dyes for use in difference gel electrophoresis detection experiments.

6. I believe that Dr. Waggoner and I acted with due diligence from a date prior to July 1993 and continuing at least through the filing of the subject application.

7. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or document or any registration resulting therefrom.

Date 12-17-2007

Jonathan S. Minden

[redacted]

CARNEGIE MELLON UNIVERSITY
PITTSBURGH, PENNSYLVANIA 15213

# DISCLOSURE OF INVENTION

1. Title of Invention: Difference Gel Electrophoresis

2. Inventors:

| | | |
|---|---|---|
| a. Jonathan Minden | [signature] | [redacted] |
| name | signature | date |
| Biological Sciences | 268-2669 | 90 |
| department | phone | % of contribution |
| b. Alan Waggoner | [signature] | [redacted] |
| | signature | date |
| Biological Sciences | 268-3459 | 10 |
| department | phone | % of contribution |

3. Brief description of invention. How does this invention relate to new processes, machines, compositions of matter, manufactures, etc? (please feel free to use additional sheets to elaborate and to attach sketches, drawings, photographs and other materials that help illustrate the description)

Cells are composed of complex mixtures of proteins, nucleic acids (RNA and DNA), and a wide variety of other compounds. An essential part of studying cells is to be able to detect or monitor differences in the protein or nucleic acid composition between different cell types, cell states or between normal and abnormal cells. We have designed and synthesized a new class of fluorescent dyes that will allow researchers and diagnosticians to detect alterations in proteins or nucleic acid composition in extracts from different cell types. .These dyes designed as matched pairs where each pair is similar with respect to molecular mass, ionic and pH characteristics, and chemical reactivity for covalent attachment to proteins and nucleic acids. The matched pairs will differ only in their spectral characteristics. The nucleus of the matched pairs is the cyanine dye molecule, where two functionalized indole rings are connected via a polyene linker. By varying the length of the linker, one can alter the fluorescence spectrum of the dye. Figure 1 details the structure and the array of possible functional groups that can be used to make different derivatives.

[redacted]

A typical application for a matched pair designed for protein modification is as follows (see figure 2 for a diagram of the procedure): the matched pairs are designated as Propyl-Cy3-NHS, which has a red fluorescence, and Methyl-Cy5-NHS, which has a blue fluorescence. The protein samples may come from cell lines where one line is normal (or wild-type) and the other is cancerous. The normal cell extract is labelled with Propyl-Cy3-NHS and the cancer line is labelled with Methyl-Cy5-NHS. The dyes become covalently linked to the lysine residues of all the proteins in each extract. The conditions are such that all possible lysines are derivatized. The Cy3 and Cy5 derivatized extracts are then mixed together and analyzed by either single dimension sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) or by two dimensional gel electrophoresis (2D-PAGE). The most sensitive method for separating complex protein mixtures is by 2D-PAGE, where one can routinely identify more than 2,000 individual protein "spots". The gels will then be analyzed by a two wavelength fluorescence gel scanner. Proteins that are common to both normal and cancerous cells will migrate on the gel as a single spot with both red and blue fluorescent components. Proteins in the two populations that differ either as a result of loss of expression or changes in post-translational modifications will not comigrate and can be detected as red and blue spots the do not contain the matched pair counterpart . Gel analysis is intended to be completely automated such that a computer will identify the protein differences. In addition, the dyes can be designed to react with different protein side chains as well as nucleic acids. We believe that these dyes represent a significant improvement in our ability to detect minor differences in complex protein and nucleic acid mixtures.

4. External Sponsor (Corporation, Government Agency, etc.):

[redacted]

5. Internal Sponsor (Departmental Research Funds, etc.):

None

6. Contract(s) or Grant Number(s):
(your departmental administrator may be of assistance in identifying your funding sources.)

7. State first date of:
   a. Conception - [redacted]
   b. Sketch or drawing - [redacted]
   c. Written description - [redacted]

[redacted]

d. Completion of working model (or operational process) - [redacted]

8. State first date of:
   a. Disclosure to other (non-CMU employees) - none
   b. Printed publication - none
   c. Oral disclosure (e.g. seminars, conferences, etc.) - none
   d. Use for profit - none
   e. Offer of sale - none

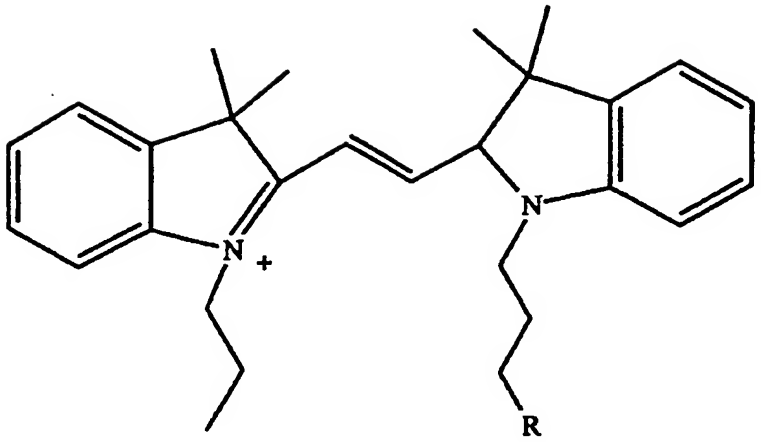
9. What makes this invention novel? How does the invention differ from present technology? What problems does it solve, or what advantages does it possess?

The present technology requires that different samples be run on separate gels and compared by visual means. There are commercially available electrophoresis systems that require extreme precision during gel fabrication and electrophoresis. In spite of these exacting standards, no two gels are identical- the pH gradients may not be perfectly linear or the electrophoresis conditions may be slightly different. Computer software for automated alignment of different gels is available. However, these software packages are all based on linear expansion or contraction of one or both dimensions. The software cannot adjust for local distortions in the gel. Our proposed system does not require computerized alignment schemes because any perturbations in the gel or electrophoresis conditions is internally controlled by have both the test sample and the control run on the same gel. Therefore eliminating the need for the expensive and meticulously controlled 2D-PAGE systems.

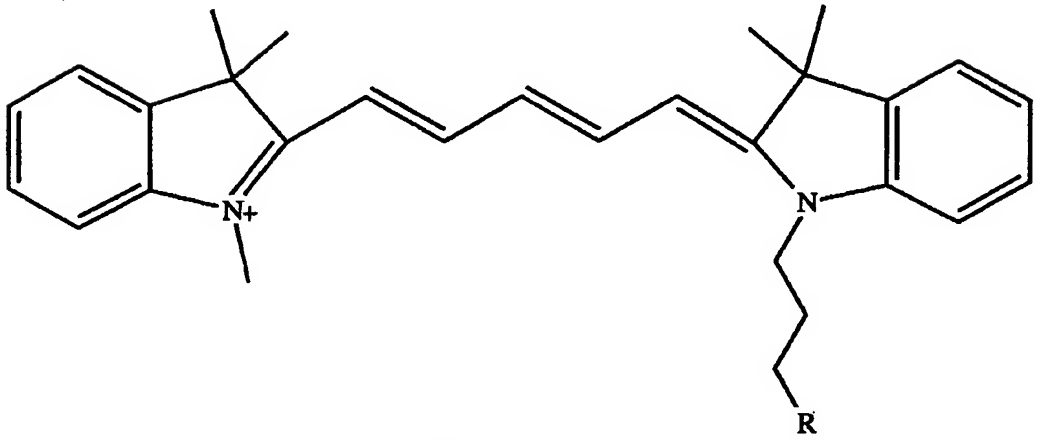
10. What are the present and future uses, applications and advantages of this invention:

In terms of protein analysis alone, one could use this matched-pair fluorescence tagging protocol to detect differences between cancer and normal cells, cells at different phases of the cell cycle, or at different stages of development. As for my own research in developmental biology, we would be able to detect the protein differences between cells destined to be part of one structure or another. We could also monitor how cells respond to a variety of stimuli or drugs. All these events that alter cellular behavior could be easily detected without the need and expense of high precision 2D-PAGE systems.

11. What are the disadvantages or limitations of this invention?




Propyl-Cy3




Methyl-Cy5

R = N-hydroxy succinimidly ester, iodo acetamide, amino, or carboxyl

Figure 1

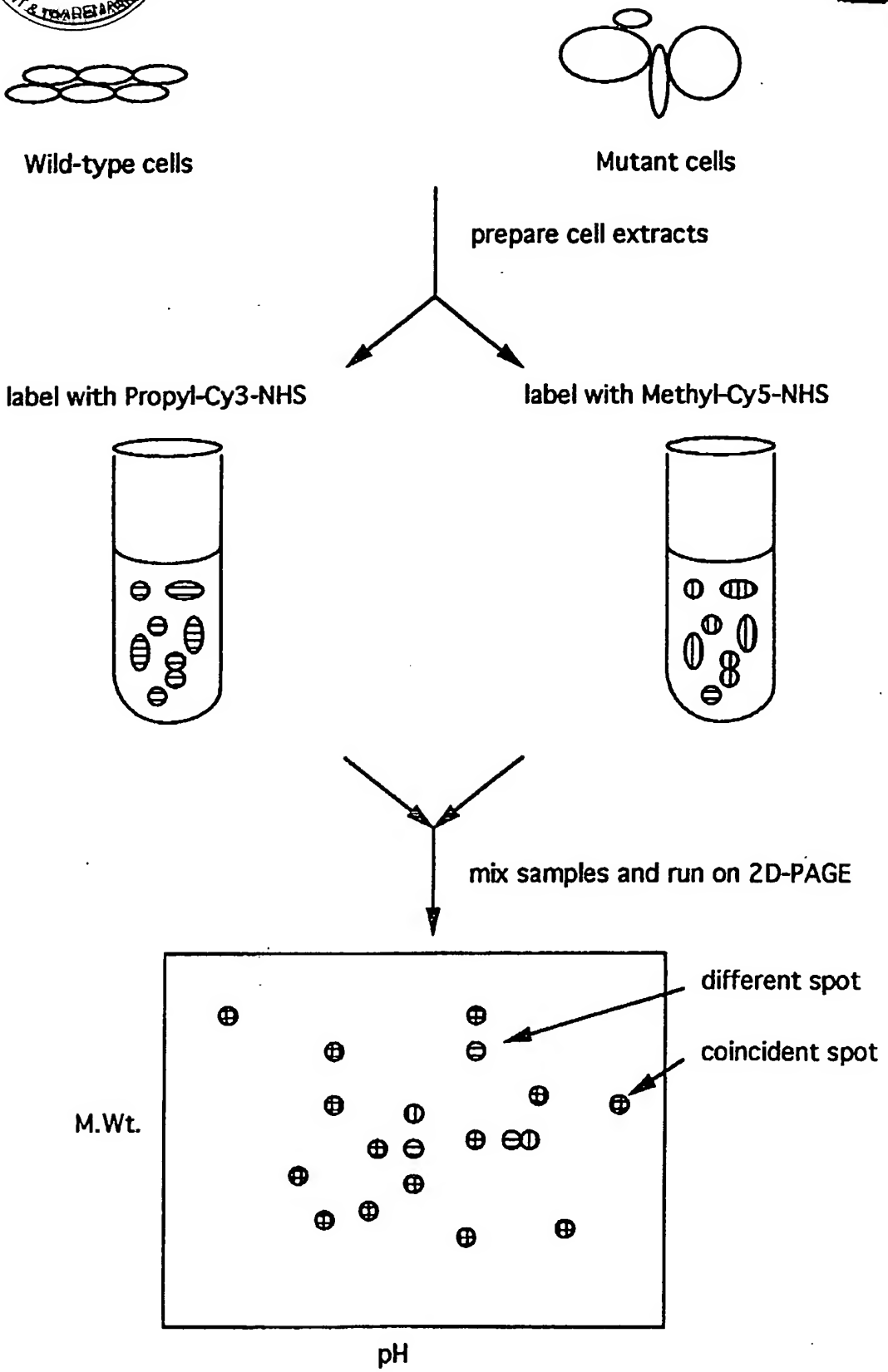



Figure 2

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# PREPARATION OF Cy5:

RECIPE FROM 6/20/92:

CB MIXED (4.8g) OF 

~0.035 MOLES (6.9g) OF 6-BROMOHEXANOIC ACID

40 ml OF 1,2-DICHLOROBENZENE

THE 3 NECKED FLASK CONTAINING THE ABOVE WAS HEATED TO ~160°C UNDER REFLUX WITH CONTINUOUS STIRRING & FLUSHED WITH $N_2$ OVERNIGHT.

---

TO AN EPPENDORF TUBE ~~I~~ ADDED 200µl OF CONC. 9-ϕ & 700µl OF 0.1M $Na_2CO_3$ / 1mM EDTA. THIS WAS SPUN DOWN 3 TIMES & RESUSPENDED IN 900µl $Na_2CO_3$.

TO 300µl OF ϕ SUSPENDED IN $Na_2CO_3$, 600µl OF TRICHLOROACETIC ACID (100g TCA DISSOLVED IN 100 ml $2H_2O$) SOLN. WAS ADDED AFTER THE ϕ WERE BOILED & SHEARED USING A 23G NEEDLE. (FROM SOLN. AWAY 10% TCA)

ALSO CARRIED OUT TCA PROTEIN PRECIPITATION AS ABOVE BUT WITHOUT SHEARING OR BOILING. FLAKY WHITISH CLUMPS APPEARED IN BOTH SAMPLES AFTER THE ADDITION OF TCA.

AFTER THE ADDITION OF TCA TO BOTH SAMPLES, THEY WERE LEFT TO SIT ON ICE FOR 20 MIN. & THEN SPUN IN THE MICROCENTRIFUGE FOR 30 MIN.

THE SAMPLES WERE THEN RESUSPENDED TO THEIR ORIGINAL CONCENTRATION WITH 1X SDS SAMPLE BUFFER

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RECORDED BY [redacted] DATE [redacted]

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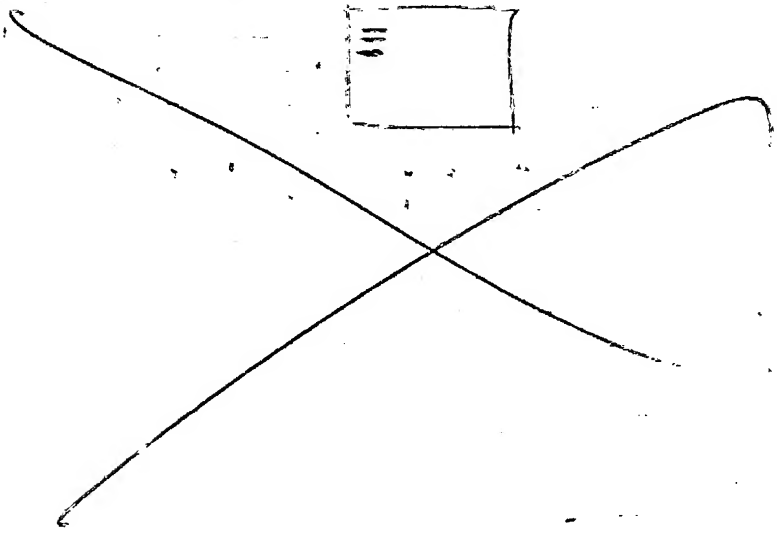
After resuspension, the samples were loaded on a 1½% mini gel. (1X electrode buffer was used) and run at 100 volts.

| | | |
|---|---|---|
| Lane 1: | 5µl | Not boiled or sheared sample |
| Lane 2: | 10µl | " " " " " |
| Lane 3: | 5µl | Boiled & sheared sample |
| Lane 4: | 10µl | " " " " |

Stained gel with Coomassie blue & destain overnight

Proper band appeared for both samples!!! yeah



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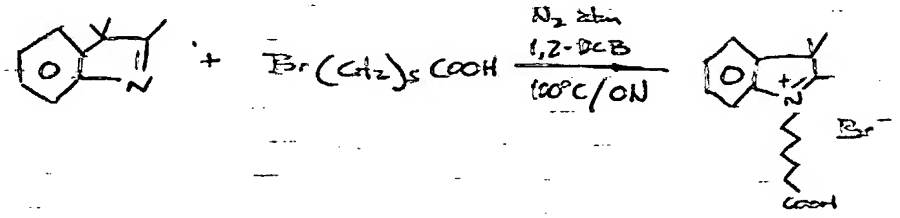
THE SYNTHESIS OF THE Cy DYE INTERMEDIATE WHICH TOOK PLACE YESTERDAY IS AS FOLLOWS:




TODAY THE RXN WAS LEFT TO COOL ON ITS OWN WITHOUT STIRRING. THE PRODUCT WAS A DARK PURPLE GUMMY MASS.

AFTER COOLING, THE 1,2-DCB WAS DECANTED OFF.

THEN, THE PRODUCT WAS WASHED WITH ~200 ml OF ETHYL ETHER AND THIS WAS POURED OFF. THE PRODUCT WAS THEN WASHED IN ~200 ml OF FRESH ETHER WHILE SCRAPPING THE SIDES WITH A SPATULA TO BREAK UP THE LUMPS. THE PRODUCT WAS STILL TOO GUMMY & TAKE-HARD TO FREE THE STIR BAR SO IT WAS LEFT TO SOAK IN THE ETHER FOR ~3 HRS. TO PREVENT THE ETHER FROM EVAPORATING & CREATING MOISTURE, THE MOUTHS OF THE FLASK WERE CAPPED TIGHTLY.
ADDED 200 ml OF FRESH ETHER & LET STIR ON.

PREPARED DYE SOLN: 5.0 mg Cy 5.18 DYE IN 50 µl DMF

RECORDED BY Chris DATE [redacted]

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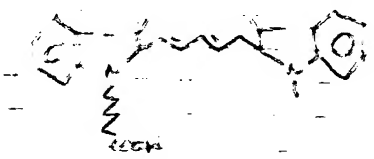
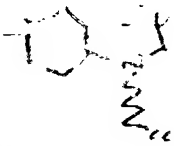
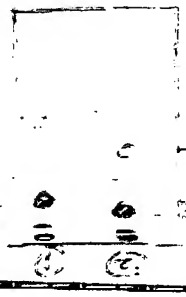
ESTIMATED 47 g OF CRUDE 

Cy5



FROM YESTERDAY AND PLACED UNDER VACUUM TO DRY OVERNIGHT.

PERFORM TLC ON PLATE. Cy5 USING 10% METHANOL IN $CHCl_3$ AS SOLVENT. TWO LANES ON PLATE. RUN. IN LANE (1) Cy5 WAS DISSOLVED IN MeOH. IN LANE (2) Cy5 WAS DISSOLVED IN $CHCl_3$. BEFORE THE PLATE WAS RUN, THE SAMPLE WAS SPIKED ON (1).




TLC SHOWED IMPURITIES

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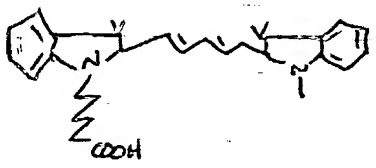
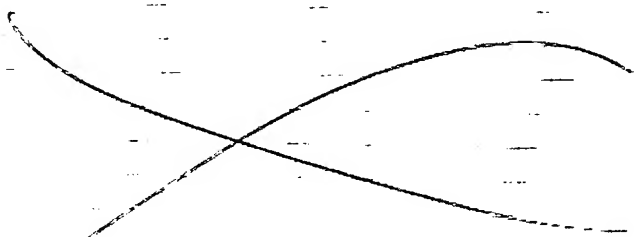
RAN A FLASH CHROMATOGRAPHY COLUMN ON CRUDE C45. FIRST 1% MeOH IN $CHCl_3$ WAS USED AS THE SOLVENT. (TOO SLOW). THEN USED 2.5% MeOH IN $CHCl_3$ (STILL TOO SLOW). NEXT, USED 5% MeOH IN $CHCl_3$.

USING TLC ANALYSIS OF THE COLLECTED FRACTIONS, THE PRODUCT WE WANTED:



WAS COLLECTED



RECORDED BY Chris

DATE [redacted]

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DATE